PATENT
2503-1228

IN THE U.S. PATENT AND TRADEMARK OFFICE

In re application of

Maria Elena FERRERO — Conf. 3886

Application No. 10/589,621 — Group 1623

Filed October 6, 2006 — Examiner L. Crane

THE USE OF O-ATP FOR THE TREATMENT OF DISEASES INVOLVING ANGIOGENESIS

DECLARATION UNDER RULE 132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Maria Elena Ferrero, hereby declare as follows:

My relevant background and experience are set forth on the attached c.v. I make this declaration in support of the present application, and to provide evidence in rebuttal of several contentions set forth in the outstanding Official Action.

In support of this position, I supervised experiments that compared the effects of oATP to that of two known compounds able to antagonize the P2X7 receptors: 1) the pyridoxal phosphate-6-azophenil-2', 4'-disulphonic acid (PPADS), a non-specific P2X7 antagonist, and 2) 1-(N, O-bis[5-isoquinolinesulphonyl]-N-methyl-L-tyrosyl)-4-phenylpiperazine (KN62), a potent P2X7 antagonist (especially at the human receptor).

The experiments used ATP (able to increase the P2X7 Receptor expression on the cells), and the P2X7 inhibitors, oATP, PPADS and KN62 at the concentrations reported in the literature, e.g.: ATP 3mM, oATP 300 microM, PPADS 50 microM and KN62 10 microM.

The first series of experiments studied the proliferation of human umbilical vein endothelial cells (HUVEC), obtained as previously described (1). The HUVEC were treated with each of the compounds for 36 hours, washed and fixed with glutaraldeide 2% in PBS. The cells were coloured with crystal violet 0.1%, washed and dried. The dye solubilisation was performed with acetic acid 10% and the absorbance was measured spectrophotometrically at 595 nm, using a microplate reader. The optical density was proportional to the number of cells. As reported in the Figure 1 (following the signature page of this declaration), cell growing number is significantly reduced by all the treatments (mean±SEM of 7 experiments), but the maximal reduction of the proliferation has been induced by oATP.

It is possible that the inhibition of the ATP-dependent pathway can induce the shift to another metabolic activity, as the glycolysis. The experiments studied if the compounds were able to induce apoptosis or necrosis of the HUVEC, by staining the cells with annexin V and propidium iodide (see the following description). The block of the HUVEC proliferation is further

evidenced by the absence of cell apoptosis due to the treatment of the cells with the tested compounds, as reported in the Figure 2 that follows the signature page of this declaration (the results are representative of 5 experiments). Only the treatment with ATP was able to induce about 4.5% PI positive, e.g. necrotic cells (data not shown).

In addition, oATP was cytofluorometrically assayed for its ability to modulate the expression of TNFalpha receptors on endothelial cells. As reported in the Figure 2a (attached after the signature page of this declaration), the use of human TNFalpha (100U/ml) improved the expression of the TNFalpha receptor 1 (TNFR1) on HUVEC. The addition of oATP alone did not modify such expression. However, oATP was able to downregulate the the TNFalpha-induced increased expression of TNFR1.

The second series of experiments studied the differences in the apoptosis induction on human promyelocytic leukaemia HL60 cells by ATP and by the inhibitors of theP2X7 receptors. Apoptotic cells and necrotic cells were analyzed by staining the cells with annexin V and propidium iodide (PI) (BD Pharmingen apoptosis kit, San Diego, CA). Briefly, an aliquot of 105 cells was incubated with annexin V-fluorescein isothiocyanate (FITC) and PI for 15 minutes at room temperature in the dark. The cells were immediately analyzed by FACScalibur (Becton Dickinson, Heidelberg, Germany). The emission/excitation wavelengths were

530/488 nm for annexin V FITC (FL1) and 640 nm/488 nm for PI (FL2). The necrotic cells were annexin V- and PI- positive, whereas apoptotic cells were annexin V-positive and PI-negative. The percentage of cells stained in each quadrant was quantified using the cellQuest software (BD Bioscience, San Jose, CA). The data, reported in the Figure 3 (attached after the signature page of this declaration), and representative of 5 experiments, show that oATP was able to induce cell apoptosis at better extent with respect to PPADS and KN62 and such effect was dependent on the concentration of oATP (data not shown). The combined use of ATP with one of the P2X7 inhibitors increased the number of necrotic cells. Figure 4 (attached after the signature page of this declaration) summarizes the effects of the different treatments on the apoptosis/necrosis induction in the HL60 cells.

Reference

1) Ferrero E, Villa A, Ferrero ME, Toninelli E, Bender JR, Pardi R, Zocchi MR. Tumor necrosis factor alpha-induced vascular leakage involves PECAM1 phosphorylation. Cancer Res. 1996 Jul 15;56(14):3211-5.

The undersigned declare further that all statements made herein of their own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Maria Elena Ferrero

January 8, 2009

Maria Elena FERRERO

Date

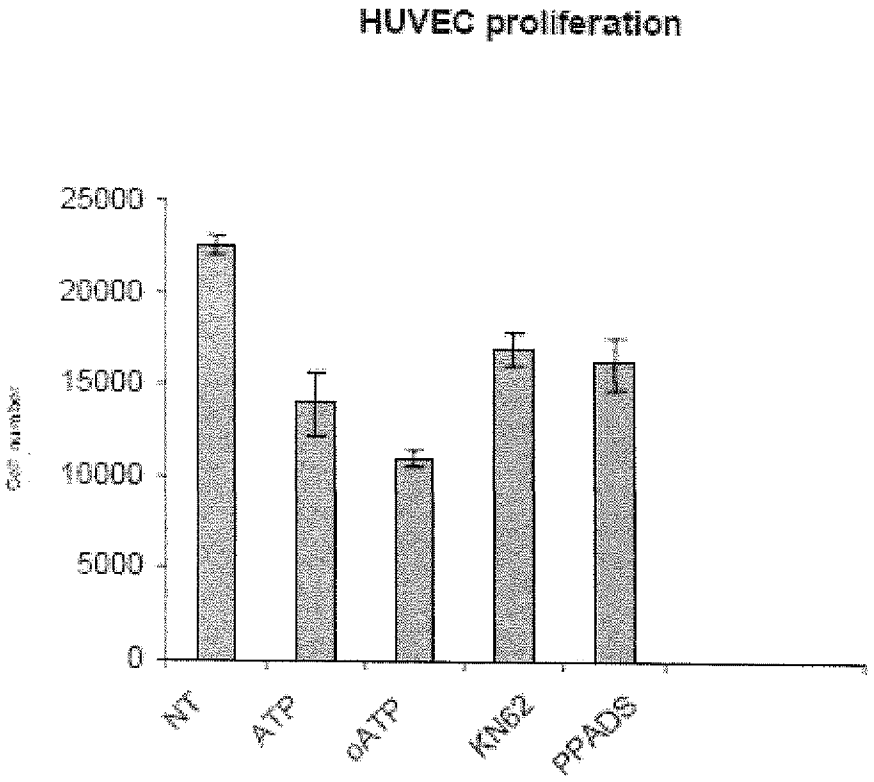
HUVEC proliferation
25000
20000
15000
10000
5000
0
NT
ATP
oATP
KN62
PPADS

Figure 1

Study of apoptosis induction on HUVEC by different treatments

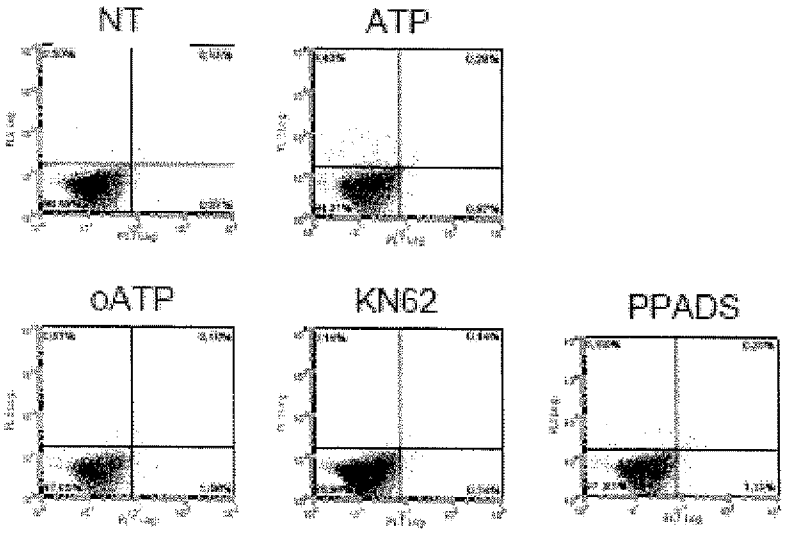

Figure 2

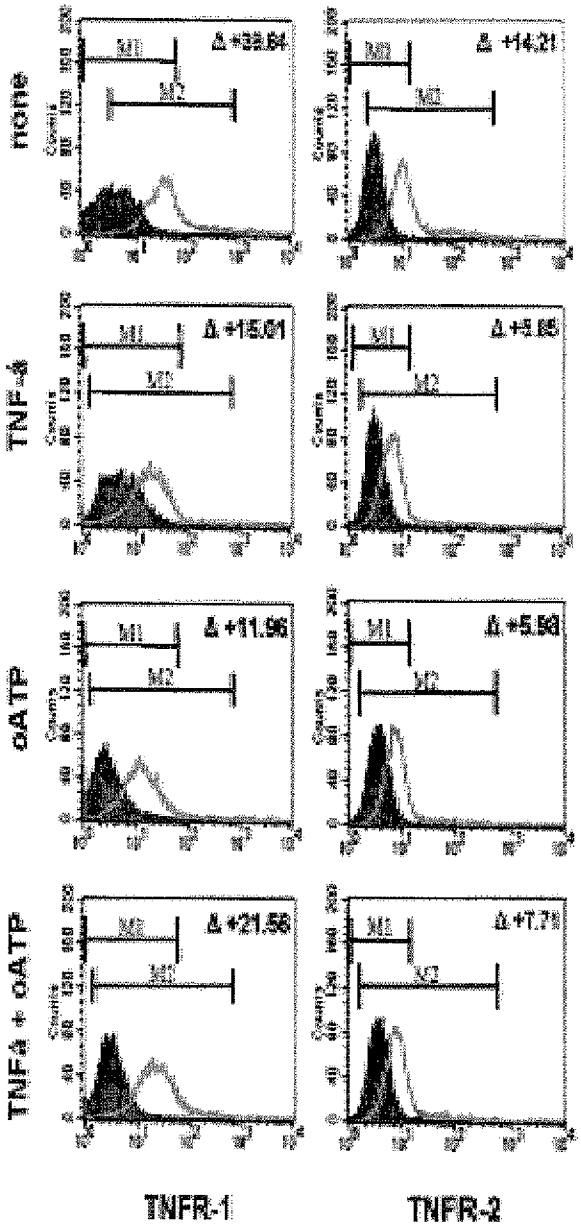

Figure 2a

Study of apoptosis induction on HL60

by different treatments

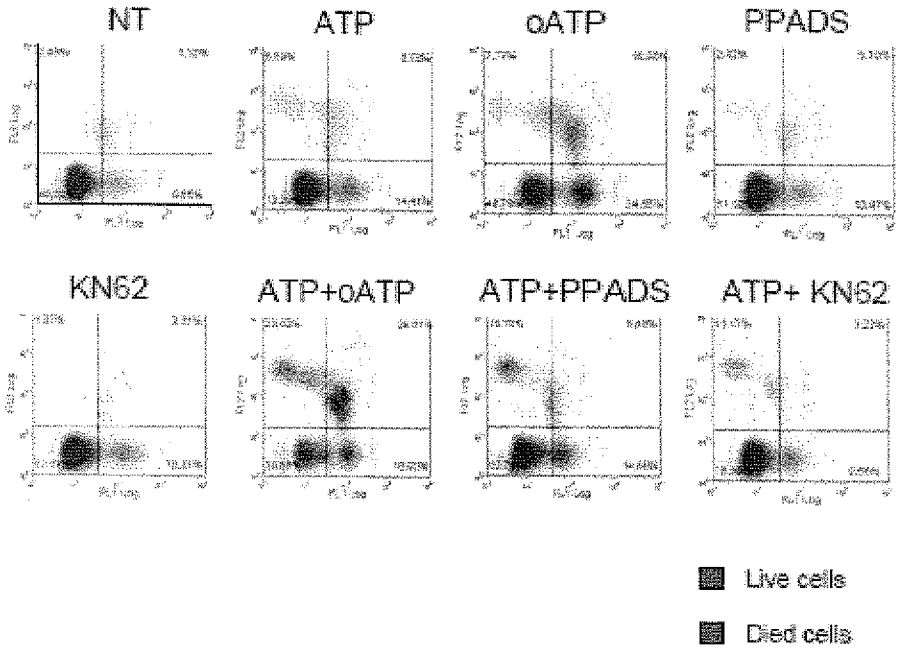

Figure 3

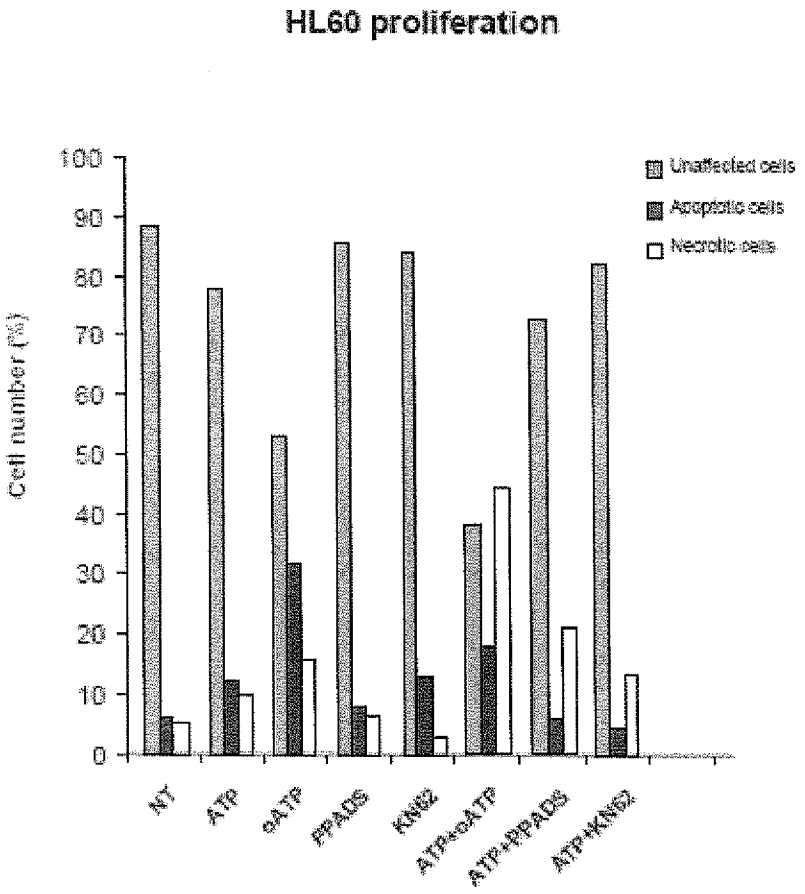
HL60 proliferation
Cell number (%)
100
90
80
70
60
50
40
30
20
10
0
Unaffected cells
Apoptotic cells
Necrotic cells
NT
ATP
oATP
PPADS
KN62
ATP+oATP
ATP+PPADS
ATP+KN62

Figure 4

CURRICULUM VITAE - (MARIA ELENA FERRERO)

PERSONAL

Citizenship, Italian.

EDUCATION

High School Diploma (Maturità Classica),

1980-M.D. (110 laude/110 votes) Medicine and Surgery, University of Milan,

1984-Specialization (70 laude/70 votes) in Anesthesia and Ranimation.

EXPERIENCE

1985 Assistant Professor of General Pathology, Institute of General Pathology, University of Milan, Italy

1990 Associate Professor of General Pathology

2003 Full Professor of General Pathology and Pathophysiology

MEMBERSHIP

Member Biochemical Society of London

Member, European Society for Organ Transplantations

Mamber of Italian Society of Physiopathologists

PUBLICATIONS

Author of 138 publications

Author of 119 abstracts presented at International and National Congresses